PriMera Scientific Engineering
Volume 1 Issue 2 October 2022
ISSN: 2834-2550

PriMera Scientific Publications

Characterization Curve of Nutrition Protein in Milk by HPLC and Its Quality Control Thereof

Type: Research Article
Received: September 21, 2022
Published: October 15, 2022

Citation:
Xindu Geng., et al. "Characterization Curve of Nutrition Protein in Milk by HPLC and Its Quality Control Thereof". PriMera Scientific Engineering 1.2 (2022): 05-14.

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Abstract

The nutrition value of milks is commonly evaluated by determining the total amount of nitrogen, amino acids, and proteins. Because of all these methods only base on the determination of a single parameter, lacking high selectivity, a lot of problems have been met. The presentation reports a highly selective method for the quality control of bovine milk by determining the relative amount of five proteins in milks by reversed-phase liquid chromatography. The fingerprint of main nutrition proteins comprising of five regions of bovine milk is firstly established and then it is characterized by a so-called as "characterization curve of nutrition protein, CCNP" which is drawn basing on the relative amount of the peak area of the five group proteins. Any significant changes in the profile of the CCNP qualitatively indicates the existence of some additives in milk, while the magnitude of the deviation from the standard CCNP is the measurement of the changes in the content of each nutrition proteins in milks. With separately adding eve's milk, soybean milk, and water into a bovine milk, these additives could be quantitatively determined with the RSD below 5% by the presented method.

Keywords: Preventive Medicine; Milk protein; Nutrition proteins; Health informatics; Medication management; HPLC

Introduction

The safety of dairy products relates to human health and thus it has been paid much attention by scientist and government officer in the world. Milk is a biologically complex fluid and the main component of bovine milk is approximately 90% water, and other components, such as proteins, fat, lactose and inorganic compounds. However, only proteins provide a great nutritional value, in which caseins accounts of 80% of the milk protein. Caseins in bovine milk consist of four different caseins as: α_{s1}-, α_{s2}-, β-, and κ-caseins, while the whey or bovine serum proteins only accounts of 20% in milk proteins [1]. The whey protein resoluble, and mainly including β-lactoglobulin (β-Lg), α-lactalbumin (a-La), and bovine serum albumin (BSA) and immunoglobulin (IGg).

Milk quality involves both of the content of nutrition proteins and authentication [2, 3]. Many methods are employed to analyze dairy products, such as the determination of total nitrogen [1], total amino acid, and total proteins. Each has its own merits, but also has some limitations even very serious problem. For example, the nitrogen sourcing from any kinds of nitrogenous compound, such as melamine and amino acids from the digesting the deleterious leather, as well total proteins from the extracts from very cheaper plants and animals which are harmful to human health totally can be not distinguished from that sourcing from the digesting nitrogen, amino acids, and proteins from milk, respectively. The shortage of the analytical method of milks used to be adopted by some illegal persons to add some additives which was mentioned above into bovine milks to occur very serious "poison milk event". Scientists have been developed many methods to solve this problem, capillary electrophoresis (CE) can separate simultaneously determine each fraction of the caseins, and/or whey protein. It has advantage of rapid, automated separations with on-line detection [4, 5], however, it requires very small size of sample but high concentration, and the experimental reproducibility is also not very good. Gel electrophoresis and isoelectric focusing also exit similar disadvantages [6].

Fingerprint technique is an effective way to systemically characterize and evaluate compositions of samples, which appears to offer a more logical approach for quality control. The fingerprint of peptides degraded from proteins [7-9] and active ingredients in traditional Chinese medicine [10-12] has been widely used. It has the characteristics of systematist, selectivity, reproducibility and high stability [13] and is usually done by qualitatively comparing the similarity between standard and sample. However, at most cases, only one component of the fingerprint is selected as an internal standard and employed to do quantitative determination. The complexity of ingredient in milk is somehow similar to traditional Chinese medicine which has a stable component and fixed relative content, providing a favorable condition for developing a new determination method of proteins in milk by means of the fingerprint.

High-performance liquid chromatography (HPLC) is currently applied to separate proteins and determine multi-component substances simultaneously. It is capable of simultaneous separation and quantification, with high resolution and reproducibility, and has been used in the development of the fingerprint method of complex materials.

The objective of our study is to establish a fingerprint consists of five nutrition proteins in bovine milk by HPLC for identifying bovine milk firstly, subsequently the established fingerprint is converted a "characterization curve of nutrition protein" by taking the total amount of whey consisting of three fractions as self-internal standard. The fitness between the standard milk and sample reveals the quality control of milk, and the deviation extent is employed to its quantitative evaluation. The method was validated by quantifying the amount of additives, including water, in bovine milk. In comparison with the conventional methods basing on one the measurement of one parameter, the presented method basing on the CCNP consisting of five parameters is not only much more exact and reliable, but also prevent from the introduce of any harmful additives into original milks by illegal persons. The presented method is expected to employ to test the quality of other milks and/or milk products.

Materials and Methods

Instruments and Reagents

The HPLC equipment (Shimadzu, Japan) consisted of two LC-20AT pumps, 7752i manual injector with a 50-μl loop, SCL-10Avp controller, SPD-M10Avp photodiode-array detector. The Class-VP 6.14 software (Shimadzu) was used for data acquisition and processing. High speed centrifuge (SORVALL, USA). pH meter (PB-10, Sartorius, Germany). Caseins (α-CN, β-CN, κ-CN) were purchased from

Sigma ((Sigma–Aldrich, St. Louis, MO, USA). Stationary phase, RPLC- C4, C8, C18 (Particle size, 2 μm; pore size: 3 0nm) was bought from RPLC-C18 (2μm, 20nm. All of the selective packings were packed into stainless steel tube (150mm×4.6mm. I.D) under 5.000 PSI in our Lab.

Guanidine hydrochloride (GaHCl, purity > 99.5%) and DL-Dithiothreitol (DDT, >98%) were obtained from Fluka (Buchs, Switzerland). Sodium citrate (analytical grade, Tianjing Hongyei Chem. Co., Tianjin, China, bis-trisbuffer (analytical grade, Kehao Biotechnology Co., Xi'an, China), acetonitrile (ACN; HPLC grade, Fisher Scientific, Pittsburgh, PA), trifluoroacetic acid (TFA; HPLC grade) were from BDH (Poole, UK). Ultrapure water (18.2MΩ•cm, cascadaTMLS, Palleoporatio) was obtained in the laboratory.

Mobile phase: Solution A, H_2O+0.1%TFA (v/v); Solution B: ACN +0.1%TFA (v/v).

Preparation of bovine milk

Denaturant solution [15]

The aqueous salt solution containing 0.1 M Bis-Tris buffer, 6.0 M GaHCl, 5.3 mM sodium citrate, and 19.0 mM DTT is adjusted pH 7 and then storage in refrigerator at 4°C. It is valid one week.

Preparation of 10 mg/mL of protein standard solution

The standard nutrition casein (α-CN, β-CN, κ-CN) were separated weighed and then dissolved in 3 mL denaturant solution with slowly shaken up to avoid from forming air bubble in the solution. After dissolving, it was filtered through 0.22 μm cellulose acetate membranes, and finally saved in refrigerator at 4°C for use.

Preparation of milk solution

9 mL denaturant solution was added into 1 mL fresh bovine milk in an ultra-centrifugation tube (in polycarbonate) and was shaken slowly to avoid from forming air bubble in the solution. After cultivating the solution for 20 min at room temperature, the supernatant then was centrifuged at 20,000 rpm (51248 g) for 2 min at 4°C. The isolated fat which forms a layer on the solution top was removed and the rest solution was filtered through a 0.22 μm cellulose acetate membranes and finally was reserved at 4°C for determination.

If a frozen milk is required to test, it firstly needs to be thawed at room temperature for 2 hours before the foregoing pre-treatment. During thawing, some milky solid, which may adhibit the vessel wall, must dissolve through vibrating.

Result and Discussion

Methodology consideration

Although scientists have worked as hard as their best to completely separate each of the main nutritional proteins of milk by HPLC, it has not been seen such a successful reported yet [16-18]. Fortunately, fingerprint does not require each component to be completely separated. The current separation efficiency of milk proteins is enough for fingerprint investigation. However, without baseline separation, it is difficult to do quantitative determination of each of the casein proteins, neither by peak height nor peak area by this way.

Even though one of several chromatographic peaks can be completely separated from other peaks and, as usually, the specific peak can be selected as a marker to evaluate the nutrition value of milk, other problems may meet. If some cheap, even harmful proteins or small solutes which have the same retention time as that as the selected specific peak, or as mentioned in introduction section, these harmful substances are added in the milk sample, a wrong result will be definitely obtained.

Thus, how to quantify each nutrition protein which can be not completely separated by HPLC is really a challenge to scientists. Fortunately, our purpose is to comprehensively evaluate the nutrition value of a milk, neither to determine total amount of the existed nutrition proteins, nor to quantify each of them nutrition. So long as the span and distribution of the peaks of the fingerprint is large and homogenous enough as possible in the chromatogram of protein separation of milk and a suitable internal standard is selected, the relative peak area of each component which builds the fingerprint can be easily converted into a characterization curve of main

nutrition of milk. This curve is possible to become the comprehensive evaluation of milk quality, not only the qualitative identification, but also quantitative evaluation.

Because of this, any non-protein nutrition substances which form chromatographic peak, they can be easily found either place on some spaces between any adjacent two nutrition peaks from the obtained fingerprint, or overlap the peaks of selected main nutrition from the characterization curve. Any positive deviations from the characterization curve indicate some additives to be introduced into the milk sample, on a contrary, any negative deviations from the curve show the milk sample was diluted, or water was added.

Stationary phase

According to the classically chromatographic dynamics theory, a higher efficiency of stationary phase is obtained from smaller particle size than bigger one, and a longer column than a shorter column. RPLC in HPLC is recognized to have the highest resolution and thus it should be selected as the stationary phase. The chain length of RPLC stationary phase was reported to affect the selectivity of various substances [19]. Both high efficiency and high selectivity are required to test with the separation of nutrition proteins.

RP-C4, RP-C8, and RP-C18 stationary phase (particle size 2μm and pore size 30nm) were employed to test the column efficiency. As usually, column efficiency is expressed with the number of theoretical plates (NTP) with small solutes, such as toluene and naphthalene et al, and then they were done the selectivity of main nutrition proteins existed in bovine milk. With naphthalene as reference solute, the NTP was 34, 213/m for RP-C4, 74,573/m for RP-C8, and 58,453/m for RP-C18, respectively. Obviously, RP-C8 shows the highest efficiency among the three columns. However, when the three columns were applied to separate the main nutrition proteins in bovine milk, as shown in Figure 1, the selectivity of both RP-C4 and RP-C18 shows a comparable, but they are much better than RP-C8. In comparing to RP-C4, RP-C18 is more popular, cheaper, so we finally chose RP-C18 as the employed stationary phase.

Two RP-C18 stationary phases having the same pore size 30 nm but with 2 μm and 3 μm pore sizes were also selected to do the same separation of the nutrition proteins of the same bovine milk, a comparable result was obtained (figure not shown here), but in comparison with the 3μm particle size, the column from 2 μm RP-C18 stationary phase was found to exhibit much higher back pressure, being unfavorable fast separation. Hence, 3 μm RP-C18 was chose in this investigation.

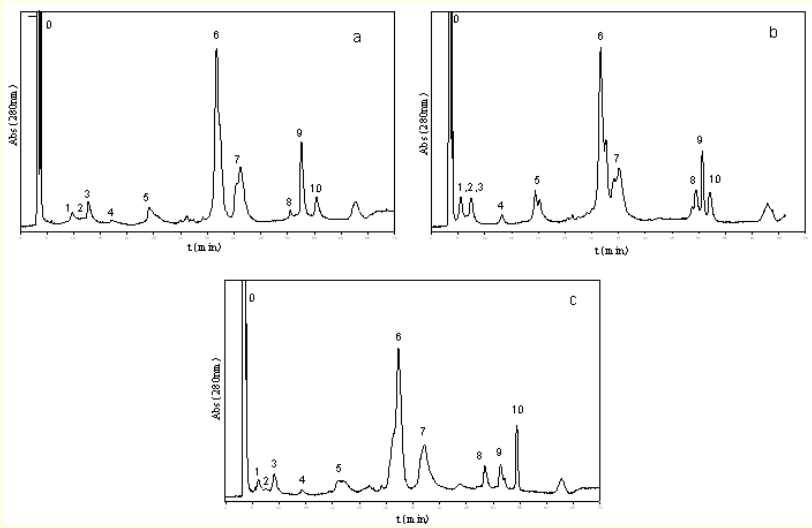


Figure 1: Chromatograms of bovine milk proteins by RPLC stationary phase (particle size, 2μm, pore size, 30nm) with different chain lengths. (a), RP-C; (b) RP-C8; (c), RP-C18. Solution A: H_2O + 0.1% TFA; solution B: ACN+0.1%TFA; linear gradient: 32% to 46% B in 50 min at 1 $mLmin^{-1}$; detection at 280 nm. Sample size: 40μL Peaks: 0, solvent; 1-4, κ-CN; 5, α_{s2}-CN; 6, α_{s1}-CN; 7, β-CN; 8-10, Whey.

Qualitative and quantitative methods of nutrition proteins in bovine milk

The fingerprint of bovine milk

Figure 2 shows the chromatogram of the standard nutrition proteins in bovine milk under an optimization conditions. According to the retention value from small to large, the whole chromatogram can be divided into five parts. Part A consists of the three peaks of κ-CN; part B denotes the peak of α_{s2}-CN; part C is the peak of α_{s1}-CN; part D stands by the peak of β-CN; and part E is the peaks of Whey, which includes α-La, β-Lg, BSA and IgG. It can be seen that, part A and part C are the sum of several nutrition proteins, which can't be completely separated, while part B and part D separately represent their single proteins, respectively. Although part E includes several whey proteins, which are not considered to be the feature nutrition proteins of milk, the completely separated four peaks also perform a part of the characterization of milk proteins. Thus the five parts of A, B, C, D, and E build up the fingerprint of milk proteins.

Any different profile from the fingerprint shown in Fig. 2 indicates the obtained chromatogram not to an original bovine milk. The existence of any extra peaks indicates some foreign substances to be introduced into the original bovine milk. However, if only one of some substances has the same retention as that of anyone of the five peak parts, it can be not identified only by the profile of the obtained fingerprint.

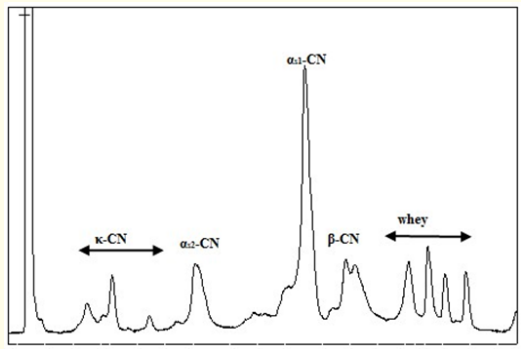


Figure 2: Five parts of fingerprint of proteins in bovine milk Column: 3μm RP-C18(150mm×4.6mm I.D). The experiment condition. protein standards: A: κ-CN; B: α_{s2}-CN; C: α_{s1}-CN; D: β-CN; E: Whey.

"Characterization curve of the nutrition proteins" (CCBM) in bovine milk

In order to exclusive the inferences from any additives having as the same retention as anyone of milk nutrition proteins, the content of both of the corresponding nutrition protein in reference milk and milk sample should be determined together. Although it certainly increases expenses and time consuming, because the additives are usually unknown, the obtaining result is only qualitative. Based on the elucidation in the section 3.1, by taking one of the five peak areas of the fingerprint shown in Fig. 2 as the internal standard, i.e. as a unity, other four area ratios $R_{(X/E}$'s are obtained.

With the unity together, the total five $R_{(X/E}$'s composing a "characterization curve of nutrition protein, (CCNP)" with the plot of $R_{(X/E}$'s vs Arabic number in the order of the magnitude of their rations a as: 1 for A/E, 2 for B/E, 3 for D/E, 4 for E/E, or unity and 5 for C/E shown in Fig. 3, respectively.

An important thing is how to select the internal standard. A good internal standard should be stable and its magnitude nearby the middle value of all of the $R_{(X/E}$ values. From Fig. 2, the part E consisting of four peak areas does not meet this condition, but also shows a completely separated four wheat proteins, providing a stable internal standard. Additionally, whey is considered as an non-nutrition protein in bovine milk, the obtained CCNP by means of this manner should be sensitive to each of the nutrition component of bovine milk.

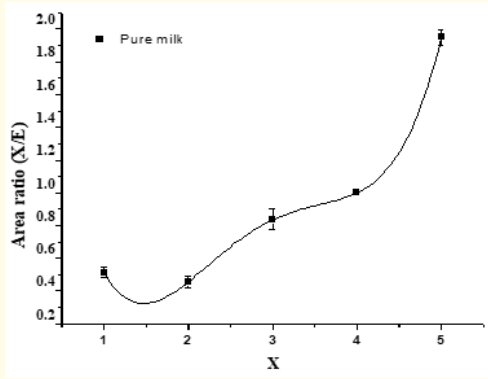
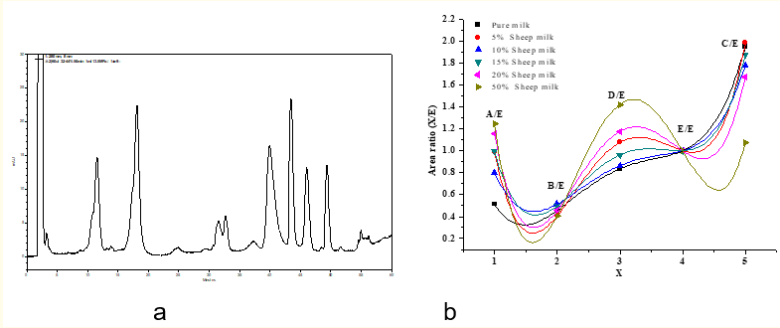


Figure 3: Characterization curve of nutrition proteins (CCNP) in bovine milk. Area parts: A: κ-CN, B: α_{s2}-CN, C: α_{s1}-CN, D: β-CN, E: Whey. Bovine milk: a milk station in Xi'an. All data were taken from Fig. 2.

Validity

In order to prove the validity of the fingerprint and the CCBM of the bovine milk, we also made the fingerprint of ewe's milk (Figure 4a) and soybean milk (Figure 4c). It can be seen from Figs. 3 and 4 that there is a great difference among the fingerprint of three kinds of milks. By taking the fingerprint of bovine milk shown in Fig. 2 as a reference here marked with blank, the changes in the R (X/E) due to adding different amounts of ewe's milks in bovine milk, as shown in Figure 4b, the ratio of A/E, B/E, C/E and D/E are totally changed by clearly seeing by eyes. Fig. 4d also shows this kind change due to the adding various amounts of soybean milk into a bovine milk. When water was added to bovine milk, the changes in the nutrition proteins can be expressed with two manners. As shown in Fig. 4e the profile of fingerprint remains invariable as the same as that shown in Fig. 3, but the obtained CCBM's of every diluted milk places below the top one sourcing from normal one. Another one is shown in Figure 4f which is expressed as the same manners, as ewe's and soybean's milks shown in Fig. 4b and 4d.



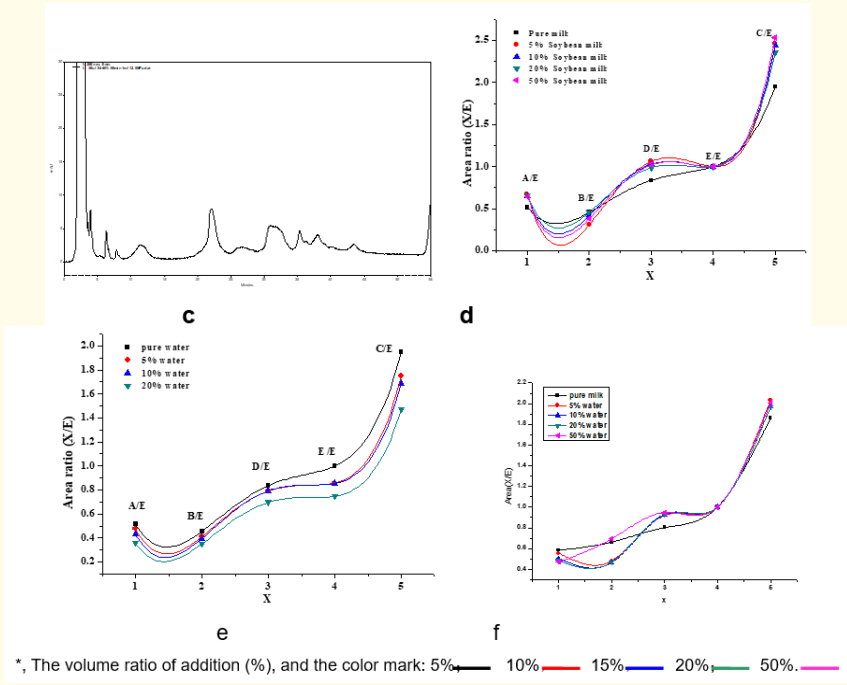


Figure 4: Comparable of CCNP between bovine milk and additives existed in bovine milk. Figs. 4a, 4c, and 4e separately denote the chromatograms of ewe's milk, soybean milk, and diluted bovine milk with water. Figs. 4b, 4d, and 4f separately represent the changes in the CCNP as different amounts of ewe's milks, soybean milks, and water was added into bovine milk. All chromatographic conditions are as the same as that shown in in Fig.3.

Figure 4 shows the possibility that the deviation value from the CCNP may be employed for the quantitative determination of each nutrition protein of bovine milk. Thus the sensitivity of the CCNP method should be firstly tested. A same original bovine milk sample was taken for four continuous analyzes. The obtained repeatability of each peak area accompanying with relative standard deviation (RSD) was listed in Table 1. It is seen that the RSD range covering from 1.7% to 6.9% and the average RSD is 3.9%.

Regions (Proteins)	***Peak area RSD (%)***
A (κ-CN)	5.2
B (α_{s2}-CN)	3.3
C (α_{s1}-CN)	1.7
D(β-CN)	6.9
E (Whey)	2.4

Table 1: Repeatability of the presented CCBM method.

Applications

We determined two groups of bovine milk in a milk monitoring station. The first group of eight samples of bovine milk has been frozen for two months at -20°C and the second group of ten fresh bovine milk samples. Fig. 5 shows the similarity of the "CCBM" of the two groups of the bovine milk samples, but some differences also exist. The quantitative determination results were listed in Table 2. The difference between the two groups may be attributed to that either between fresh and storage, or that between two sources.

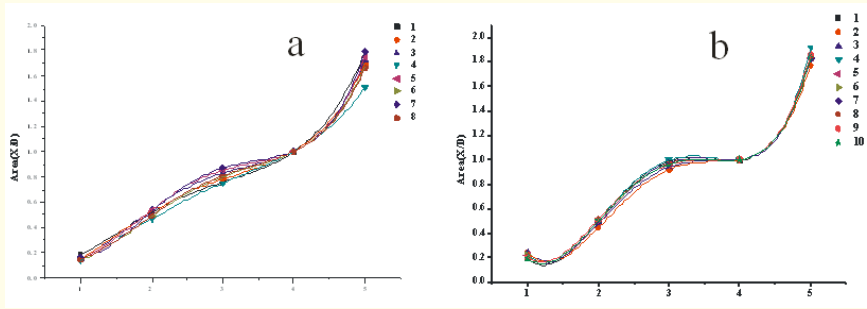


Figure 5: The "characterization curve" of the two groups of bovine milk samples, a: fresh bovine milk; b: bovine milk standing in two months in refrigerator.

Numbering	*A/E*	*B/E*	*C/E*	*D/E*	*E/E*
Set 1	5.1%	8.7%	5.4%	5.3	0.0
Set 2	3.6%	8.6%	1.9%	5.2	0.0

Table 2: Comparison of RSD two sample groups.

To find out the reason why the differences exist between the two group samples, the dynamic of the degradation of the five nutrition proteins in two commercially available bovine milks ("a" and "b") were investigated. When the two milks were stand for 8, 16, 24, and 42 hours under 30 (±1) ℃, the changes in the relative content of these proteins are shown in Fig. 6. First, it is seen that the two sets of the CCNP" of bovine milk degradation have an identical profile, qualitatively indicating the reliability of the presented CCNP method. Second, all proteins degraded, but quite different for each. From Fig. 6, the changes in the R (B/E) of α_{s2}-CN is the smallest, while the R D/E) of β-CN is the largest. All of these data were obtained from the average of continues three batches covering 7 days, and two continues repeats for every batch chromatographic separations. The standard derivation from the determination of these nutrition proteins a day and from day to day are separately shown in Table 3. By taking the standing time 0 h as an example, except the R B/E of α_{s2}-CN, the RSD of other nutrition proteins are totally less than 10%. This fact shown in Figs 5 and 6 indicates that the differences of the CCNM mainly from the standing time, but two sources.

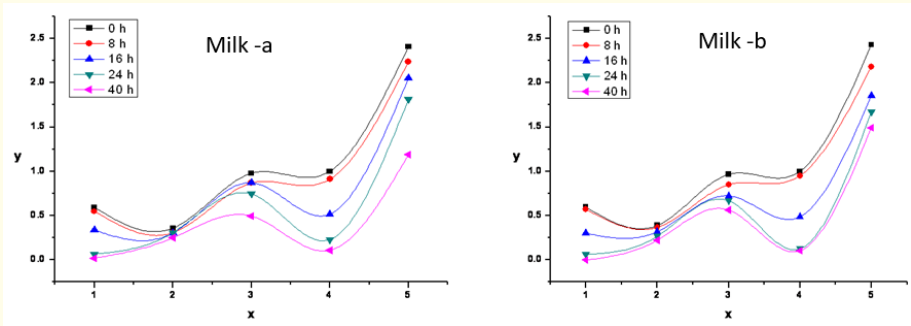
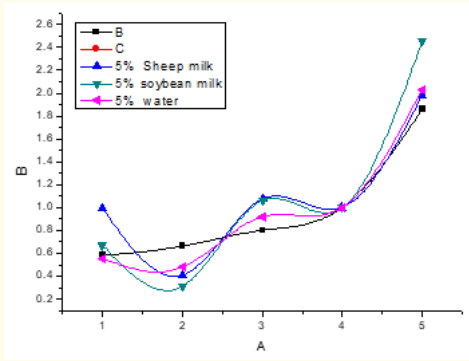


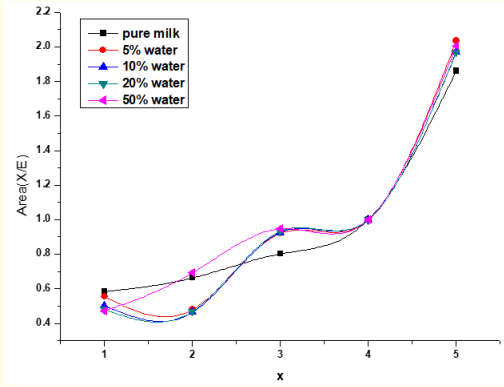
Figure 6: The dynamic CCNP of two kinds of commercially available pure milks standing from 0~40 h.

Milk	*A/E*	*B/E*	*C/E*	*D/E*	*E/E*
In a day	2.3%	11.1%	3.7%	2.7%	4.1%
Day to day	8.7%	13.7%	7.2%	10.0%	8.5%

Table 3: The RSD two kinds of milk standing 0h.



Changes in CCNP as the content of sheep milk, soybean milk, and water is 5% in bovine milk, respectively.



In conclusion, the suggested new methodology for quantitative determination of a set of nutrition proteins in milks by RP-HPLC. With the "characterization curve of nutrition protein, CCNP" of bovine milk, the changes in the relative content of any nutrition proteins in milk can be detected and quantified. The validity of this method was proved by adding eve's milk, soybean milk and water with the RSD below 5%, indicating to have high sensitivity and selectivity. In comparison to conventional method, the determination of total nitrogen, total amino acids, or total proteins, the CCNP has advantages of high reliability. It would be expected that the presented method may be employed for the quality control from the origin of milk milk collection station, quality control of milk process and final product of daily bovine, sheep, and also milk power. It may be also employed to investigate how to raise the out-put of nutrition proteins of bovine milk in farms. The most important thing is probably that the CCNP method may break down a luck mentality trying to raise up milk quality by putting some additives into the milks.

Acknowledgment

The work is supported by the Foundation of Provincial Key Laboratory of Modern Separation Science (No. 0505JS60).

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